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Effects of Hypothyroidism on Development of Nitrosomethylurea-Induced Tumors of the Mammary Gland, Thyroid Gland, and Other Tissues[1] (41379)

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Abstract. Female rats were injected with the mammary gland carcinogen *N*-nitrosomethylurea (NMU) and were made hypothyroid by treatment with either propylthiouracil (PTU) or ^{131}I. NMU (25–100 mg/kg) itself caused a dose-related increase in serum thyroxine levels. PTU (1.0–3.0 mg/100 ml of drinking water) caused a decrease in serum thyroxine and triiodothyronine levels in NMU-treated rats. At 28–31 weeks after injection of NMU alone, 9/43 rats (21%) had malignant mammary tumors. This incidence was increased to 11/30 (37% $P > 0.1$) and 13/30 (43% $P < 0.05$) in rats treated with PTU doses of 0.3 and 1.0 mg/100 ml, respectively. In contrast, the 3 mg/100 ml dose of PTU reduced the tumor incidence to 2/30 rats (7%, $0.1 > P > 0.05$) and caused a 33% decrease in final body weight. Treatment with 10 μCi of ^{131}I gave a small decrease in serum thyroxine levels in NMU-treated rats and increased the incidence of malignant mammary tumors to 11/26 rats (42%, $0.1 > P > 0.05$). In addition, PTU (0.3 mg/100 ml) plus NMU induced a 23% incidence (7/30 rats) of malignant thyroid tumors, and PTU doses of 1.0 or 3.0 mg/100 ml plus NMU led to a 100% incidence of these tumors. With the 0.3 mg/100 ml dose of PTU, there was an increase in the incidence of lung adenomas, whereas with the 3.0/100 ml dose there was a decrease. Thus, this study demonstrates that in NMU-treated rats: (1) slight hypothyroidism favors development of mammary and lung tumors, (2) severe hypothyroidism produces the opposite effect together with weight loss, and (3) the combination of PTU treatment and the carcinogen is followed by malignant thyroid tumors.

The evidence that abnormal thyroid function is associated with an increased risk of breast cancer in humans remains controversial. For example, a positive association has been noted between the incidence of endemic goiter and breast cancer in various parts of the world (1), whereas measures of thyroid function such as protein-bound iodine have been reported to be normal in women with breast cancer (2). Other studies indicate that more sensitive tests of thyroid function, e.g., circulating thyrotropin (3) or responsiveness to thyrotropin-releasing hormone (4), yield abnormal results in women with breast cancer. In part, these conflicting results may be due to the effect of the cancer on thyroid function, and they may not bear on the effect of thyroid hormones on breast cancer development. An increase in breast cancer incidence was noted among women treated with thyroid supplements (5), but it may be that the antecedent hypothyroid condition of patients, rather than the hormonal treatment, might be responsible for their increased risk (6). The situation is clearly complex, for a later epidemiologic study (7) could find no association between breast cancer incidence and the use of thyroid hormone therapy. Wallace *et al.* (7) indicated the need for further studies to clarify any possible association among women with true hypothyroidism using hormonal treatment.

Studies in laboratory animals have also yielded conflicting results (8). For example, treatment of rats with antithyroid drugs or

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^{131}I appears to decrease the yield of mammary tumors induced by 7,12-dimethylbenz (*a*)anthracene (DMBA) (8, 9). In a more recent study, there seemed to be no relationship between thyroid function and mammary tumor incidence in rats given *N*-nitrosomethylurea (NMU), but mammary tumors showed an increased rate of growth when transplanted to hyperthyroid rats (10). However, Eskin (11) has noted more mammary tumors in slightly hypothyroid rats. Since it has been proposed that NMU-induced mammary tumors represent a better model of human breast cancer than tumors induced by DMBA (12), the present study was designed to examine the effects of hypothyroidism on the development of NMU-induced mammary tumors in rats.

Materials and Methods. *Animals.* Female Fischer 344 rats were obtained from Charles River Breeding Laboratories (North Wilmington, Mass.) and were housed in a quarantine area in plastic cages (three rats/cage) with hardwood chip bedding for 2 weeks before any treatments. Routine tests were performed on randomly selected rats to certify health status prior to transfer to the holding rooms. The environment was controlled with respect to temperature (23 ± 1°), light (12-hr cycle), and humidity 42 ± 4%). The rats were fed Purina Lab Chow and water *ad libitum*.

Drugs. NMU (Starks Associates, Buffalo, N.Y.) was moistened with a few drops of 10% acetic acid and dissolved in distilled water immediately before use. Propylthiouracil (Nutritional Biochemicals, Cleveland, Ohio) was prepared fresh weekly in tap water and stored at 5° until use. ^{131}I (New England Nuclear, Boston, Mass.) was diluted in distilled water.

Assays. Thyroxine (T_4) and 3,5,3′-L-triiodothyronine (T_3) were measured by radioimmunoassays using kits obtained from Diagnostic Products Corporation, Los Angeles, California.

Treatments. Group I served as untreated controls; Group II was injected with NMU (50 mg/kg) iv at 50 days of age; Groups III, IV, and V were injected with NMU, and were given 0.3, 1.0, and 3.0 mg propylthiouracil, respectively, per 100 ml of drinking water, beginning 5 days after the NMU dose; Groups VI and VII were injected ip with 1 and 10 μCi, respectively, of ^{131}I 2 days after treatment with NMU. Rats were weighed at the start of the experiment, every other week thereafter, and palpated for mammary tumors weekly. At the time of autopsy, the number of tumors was verified by at least one additional observer.

Six weeks after treatment with NMU, randomly selected rats from each group were anesthetized with ether and blood samples were collected by suborbital puncture. Serum was separated by centrifugation and stored at −20° until assayed for thyroxine and triiodothyronine. Rats were sacrificed by carbon dioxide at 28−31 weeks after treatment with NMU. Tumor volumes were estimated by the formula used by Gullino *et al.* (12), i.e., vol = $4/3\ r^3$, where r is one-half the average of the longest and shortest diameters of each tumor. Ovarian, uterine, and adrenal weights were determined for randomly selected rats.

Histology. Mammary tumors were removed, measured, and fixed, as were select other tissues, in 10% buffered formalin solution. Tumors and normal sections of thyroid, ovaries, adrenals, pituitary, spleen, pancreas, liver, kidney, lungs, trachea, and thymus were prepared for histologic study. Sections were embedded in paraffin from which 5-μm-thick sections were cut. The slides were routinely stained with hematoxylin and eosin (H&E). Whenever necessary, extra sections were cut and stained with trichrome (Mallory's), PAS, and reticulum stains).

Statistics. Data were evaluated by Student's t-test or by χ^2 analysis (13).

Results. Six weeks after injection of 50 mg/kg of NMU, serum thyroxine levels were significantly elevated (Table I). The 100 mg/kg dose caused death of 8/12 rats and serum thyroxine levels were increased in the surviving animals.

Treatment with PTU (0.3 mg/100 ml) prevented the NMU-induced elevation of serum thyroxine levels (Table II). Larger doses of PTU (1.0 and 3.0 mg/100 ml) decreased the thyroxine and triiodothyronine levels

TABLE I. EFFECT OF *N*-NITROSOMETHYLUREA (NMU) ON SERUM THYROXINE (T_4) LEVELS

Treatment	Dose (mg/kg, iv)	No. rats	Serum T_4 (μg/100 ml)
Control	—	6	4.1 ± 0.4[a]
NMU	25	6	4.8 ± 0.3
NMU	50	6	6.0 ± 0.4*
NMU	100	4	7.0 ± 0.6*

Note. 50-day-old rats were treated with a single dose of NMU, and blood samples were collected 6 weeks later.

[a] Mean ± SEM.

* $P < 0.01$ vs control.

further. The 10 μCi dose of ^{131}I led to a slight but significant decrease in serum thyroxine levels of NMU-treated rats but did not affect triiodothyronine levels. The first palpable mammary tumors were observed at 10–12 weeks after treatment with NMU. The incidence of malignant mammary tumors at 28–31 weeks was increased in rats treated with either PTU (0.3 mg/100 ml) or ^{131}I (10 μCi) (Table II). The average volume of these tumors was decreased in rats treated with PTU (1.0 or 3.0 mg/100 ml), and the final body weights were decreased by these doses of PTU. The incidence of malignant mammary tumors was decreased in rats receiving the highest dose of PTU (3.0 mg/100 ml).

Weights of ovaries, uteri, or adrenal glands of randomly selected rats (three to six per group) were not affected by NMU or by the combination of NMU plus PTU (0.3–3.0 mg/100 ml).

The 0.3 mg/100 ml dose of PTU caused the appearance of benign and malignant thyroid tumors in NMU-treated rats (Table III, Group III). The 1.0 and 3.0 mg/100 ml doses resulted in a 100% incidence of malignant thyroid tumors in NMU-treated rats (Groups IV and V). No thyroid tumors were observed in the remaining groups. The 0.3 mg/100 ml dose of PTU increased the incidence of lung adenomas ($0.1 > P > 0.05$), whereas the higher doses had no effect (Group IV) or were inhibitory (Group V, $0.1 > P > 0.05$) with respect to the appearance of this type of tumor.

Microscopic pathology of the mammary gland. All the benign neoplasms were fibroadenomas that either occurred alone or in combination with carcinoma elsewhere in the animal.

All the malignant neoplasms were adenocarcinomas that were sometimes multiple (14 tumors in 11 animals in Group III, 16 tumors in 13 rats in Group IV, 16 tumors in 11 animals in Group VII). The adenocarcinomas also occurred in combination with fibroadenoma, thyroid carcinoma, lymphoma, or nephroblastoma of the kidney. The mammary gland carcinomas were well circumscribed and lobulated, often with a cystic component. Solid as well as papillary areas were seen. No metastases were observed.

Microscopic pathology of the thyroid gland. All the benign tumors occurred in Group III (NMU + PTU 0.3 mg/100 ml), and were follicular adenomas. They were encapsulated and made up of follicles of varying sizes. Two of the five adenomas were cytologically atypical. In three instances, the adenomas occurred in combination with carcinoma elsewhere in the gland.

The malignant thyroid tumors developed in Groups III, IV, and V (Table III). The predominant histologic pattern was follicular, or follicular mixed with papillary carcinoma. A small percentage of undifferentiated small cell tumors with focal follicular differentiation was found. Pure papillary carcinomas were rare. In 5 out of 68 cases, careful searching revealed psammoma bodies. All the tumors had capsular invasion with vascular invasion in most of the cases. Many tumors infiltrated into adjacent muscle, cartilage, and trachea. Seven animals (all in Group V) developed multifocal hematogenous micrometastasis to lung, but no metastasis to lymph nodes was observed.

Microscopic pathology of other tumors. There were four nephroblastomas of kidney (stromal nephroma), two in Group III, and two in Group VI. They consisted of predominant spindle cell sarcomatous stroma with few trapped benign appearing tubules. There were two squamous carcinomas of the skin, one in Group VI and one in Group VII. There were eight occurrences of

TABLE II. EFFECTS OF PROPYLTHIOURACIL (PTU) ON MAMMARY TUMOR DEVELOPMENT

Group treatment	N	Final body wt. (g)	Serum thyroxine[a] (μg/100 ml)	Serum Triiodo thyronine[a] (ng/100 ml)	Incidence of mammary cancer (%)	Number of malignant mammary tumors	Volume of malignant mammary tumors
I. Control	12	175[b] ± 13	4.1 ± 0.4	108 ± 4	0	0	—
II. NMU (50 mg/kg, iv)	43	173 ± 4	6.0[c] ± 0.3	116 ± 5	21	10	3.9 ± 1.5
III. NMU + PTU (0.3 mg/100 ml)	30	175 ± 3	4.4[d] ± 0.4	117 ± 4	37	14	6.7 ± 2.9
IV. NMU + PTU (1.0 mg/100 ml)	30	163[h] ± 4	2.5[e,f] ± 0.5	92[c,f] ± 3	43[g]	16	0.7[h] ± 0.4
V. NMU + PTU (3 mg/100 ml)	30	116[f,i] ± 2	0.7[f,i] ± 0.1	55[f,i] ± 5	7[h]	2	0.3[g] ± 0.1
VI. NMU + ^{131}I (1 μCi)	30	178 ± 4	5.1 ± 0.6	105 ± 6	20	6	0.9 ± 0.7
VII. NMU + ^{131}I (10 μCi)	26	180 ± 4	4.9[g] ± 0.4	112 ± 10	42[h]	16	4.1 ± 2.8

Note. Rats were injected with NMU at 50 days of age. PTU at the concentration indicated, was placed in the drinking water beginning 5 days after NMU.

[a] Blood samples (six per group) were collected 6 weeks after NMU.

[b] Mean ± SEM.

[c] $P < 0.01$ vs Group I.

[d] $P < 0.01$ vs Group II.

[e] $P < 0.05$ vs Group I.

[f] $P < 0.001$ vs Group II.

[g] $P < 0.05$ vs Group II.

[h] $0.1 > P > 0.05$ vs Group II.

[i] $P < 0.001$ vs Group I.

TABLE III. TUMOR INCIDENCE IN RATS TREATED WITH NMU AND PTU

Group	Treatment	No. rats	Tumor-bearing rats: Thyroid No. (%)	Lung No. (%)	Kidney No. (%)
I.	Control	12	0	0	0
II.	NMU (50 mg/kg, iv)	43	0	7 (16)	0
III.	NMU + PTU (0.3 mg/100 ml)[a]	30	12[b,c] (40)	12[d] (40)	2[e] (7)
IV.	NMU + PTU (1.0 mg/100 ml)	30	30[c] (100)	5 (17)	0
V.	NMU + PTU (3.0 mg/100 ml)	30	30[c] (100)	1[d] (3)	0
VI.	NMU + ^{131}I (1 μCi)	30	0	8 (27)	2[e] (7)
VII.	NMU + ^{131}I (10 μCi)	26	0	7 (27)	0

[a] Concentration of PTU in drinking water.
[b] Five of the thyroid tumors in this group were benign. The remaining thyroid tumors in this and subsequent groups were malignant.
[c] $P < 0.005$ vs Group II.
[d] $P < 0.025$ vs Group II.
[e] $0.1 > P > 0.05$ vs Group II.

lymphoma/leukemia: two per group in Groups II and IV and one per group in Groups III, V, VI, and VII.

The benign tumors included many lung adenomas, epidermal cysts of skin, endometrial polyps, and a single cortical adenoma of the adrenal. None of the animals treated with NMU alone had epidermal cysts, whereas each of the PTU or ^{131}I treated groups had one tumor of this type except for Group V which had two such tumors.

The pituitary glands appeared slightly hypertrophied in the animals which received PTU or ^{131}I. There were many large hypertrophied "chromophobic" PAS-negative cells. No tumors of pituitary were noted. The thyroid glands of the animals treated with ^{131}I showed follicles of varying sizes. The larger follicles were filled with colloid and lined by flat epithelium. The smaller follicles were lined by tall columnar epithelium with moderate nuclear irregularities. However, no mitosis or invasion, or interstitial fibrosis was noted in those glands.

Discussion. The results of this study demonstrate that slight hypothyroidism can increase the incidence of NMU-induced mammary tumors in the rat. Previous investigators (8–10) have not observed such a relationship, probably because the degree of hypothyroidism that they induced was too severe. For example, Kellen (8) reported that PTU or methimazole could reduce the incidence of mammary tumors in DMBA-treated rats. The same doses of these drugs, however, caused significant inhibition of body growth. Likewise, Jabara and Maritz used ^{131}I treatment to inhibit thyroid function and also found a decreased incidence of DMBA-induced mammary tumors (9). But, again, the ^{131}I treatment inhibited the rate of body growth. In the present study, we also found that, when hypothyroidism was severe enough to inhibit growth, tumor incidence was clearly reduced. It seems likely that this reduction is due to the well-established inhibition of tumorigenesis due to nutrition-mediated effects (14, 15).

Other investigators (11) have shown that PTU or an iodine-deficient diet can hasten the appearance of DMBA-induced mam-

mary tumors. The present results, however, differ from those of Cave *et al.* (10) who induced mammary tumors by multiple doses of NMU. Differences in experimental design preclude a direct comparison with the present study, but a single dose of NMU probably yields a clearer approach to mechanisms.

The mechanisms by which hypothyroidism might increase the incidence of mammary tumors are not clear. It is well established that hypothyroidism alters secretion of gonadotropins (15, 16) and metabolism of steroid hormones (18–20). It seems unlikely that the changes in mammary tumor incidence seen in the present study were mediated by these hormones. Another possible mechanism may involve prolactin, since both prolactin secretion (10) and the effects of prolactin on the mammary gland (21) are affected by changes in thyroid function.

The results of this study lend support to the view that slight hypothyroidism may play a role in the development of breast cancer (4). It is important to realize that this type of hypothyroidism can occur asymptomatically, and its occurrence can be established only by sensitive tests such as serum thyrotropin levels (22). In this regard, the report that women treated with thyroid hormones have a relatively high incidence of breast cancer (5) might be explained by early events in the process prior to the initiation of hormone replacement or by dosing inadequate to reconstitute hormone balance.

The present observation might also be explained by other mechanisms. For example, since the doses of ^{131}I used were not sufficient to alter serum thyroid hormone levels, it is possible that ^{131}I acted directly on the mammary gland to increase the incidence of NMU-induced tumors. Furthermore, the increase in serum thyroxine levels seen after treatment with NMU alone complicates the interpretation of this study, although the duration or permanence of the increase after a single dose of NMU is not known. Finally, extrathyroidal effects of either NMU or PTU might have contributed to the development of tumors.

It has been known for many years that goitrogens such as thiouracil favor the development of thyroid tumors induced by carcinogens such as 2-acetylaminofluorene (23, 24). The present study shows that a similar relationship exists between propylthiouracil and NMU and yielded malignant thyroid cancers. In humans, there is evidence both for (25) and against (26) the possibility of a positive correlation between the incidence of endemic goiter and thyroid cancer. We have observed that NMU treatment causes an elevation of serum thyroxine levels 6 weeks later. Since total thyroxine levels were measured, this could be due to an increase in thyroxine-binding globulin. Interestingly, Kieffer *et al.* (27) have observed that NMU treatment causes thyroiditis in the Buffalo strain of rat. Furthermore, a relationship has been noted between thyroiditis and thyroid carcinoma in humans (28).

After the completion of this study, it was reported that PTU did not influence the induction or growth of mammary tumors induced by 7,12-dimethylbenz(*a*)anthracene (DMBA) (29). The difference between these results and those of the present study may be partly due to the fact that a different carcinogen and a different strain of rat were used. Nonetheless, Goodman *et al.* (29) did observe a significant decrease in tumor incidence in rats with severe hypothyroidism.

Coupled with recent reports indicating a relationship between hypothyroidism and breast cancer in women (30, 31), the results described here suggest that more attention should be paid to the early detection and treatment of slight hypothyroidism as a means of lowering the risk of breast cancer. Furthermore, since endemic goiter may be associated with an increased incidence of breast cancer (1), providing adequate dietary iodide may be a useful complement to dietary intervention such as a reduction of total fat intake as an approach to the prevention of breast cancer (32).

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